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Thermolability of Ubiquitin-Activating Enzyme from the Mammalian Cell Cycle Mutant ts85

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Summary

Ubiquitin, a 76 residue protein, occurs in eucaryotic cells either free or covalently joined to a variety of protein species. Previous work suggested that ubiquitin may function as a signal for attack by proteinases specific for ubiquitin–protein conjugates. We show that the mouse cell line ts85, a previously isolated cell cycle mutant, is temperature-sensitive in ubiquitin–protein conjugation, and that this effect is due to the specific thermolability of the ts85 ubiquitin-activating enzyme (E1). From E1 thermoinactivation kinetics in mixed (wild-type plus ts85) extracts, and from copurification of the determinant of E1 thermolability with E1 in ubiquitin-affinity chromatography, we conclude that the determinant of E1 thermolability is contained within the E1 polypeptide. ts85 cells fail to degrade otherwise short-lived intracellular proteins at the nonpermissive temperature (accompanying paper), demonstrating that degradation of the bulk of short-lived proteins in this higher eucaryotic cell proceeds through a ubiquitin-dependent pathway. We discuss possible roles of ubiquitin-dependent pathways in DNA transactions, the cell cycle, and the heat shock response.

Introduction

Ubiquitin is a 76 residue protein abundant in apparently all eucaryotic cells. Its amino acid sequence is one of the most conserved known, being identical in humans and insects (Goldstein et al., 1975; Gavilanes et al., 1982). Ubiquitin occurs in cells either free or covalently coupled via its carboxyl terminus to ε-amino groups of lysine residues in a wide variety of intracellular protein species, the most abundant of which is apparently histone H2A (Chin et al., 1982; Atidia and Kulka, 1982). The ubiquitin–H2A semihistone (uH2A), in which the carboxyl terminus of ubiquitin is joined via an isopeptide bond to the internal lysine 119 in histone H2A (Goldknopf et al., 1975; Busch and Goldknopf, 1981; see also West and Bonner, 1980), substitutes for one or both of the nucleosomal H2A molecules in 10% to 20% of nucleosomes; ubiquitinated nucleosome species occur preferentially within transcribed chromosomal regions (Levinger and Varshavsky, 1980, 1982; Varshavsky et al., 1983). A specific factor required for ATP-dependent, nonlysosomal proteolysis in reticulocyte extracts (Ciechanover et al., 1978) was purified and shown to form multiple covalent conjugates to acceptor proteins in the presence of reticulocyte extracts (Ciechanover et al., 1980; Hershko et al., 1980). This factor was subsequently identified as ubiquitin (Wilkinson et al., 1980). The proposed mechanism of ubiquitin action in this system (see Figure 1) is through isopeptide bond formation to substrates for proteolysis; one possible role of ubiquitin is to serve as a signal for attack by proteinases specific for ubiquitin–protein conjugates (Hershko and Ciechanover, 1982).

The enzymatic pathway of ubiquitin conjugation in reticulocyte extracts has recently been elucidated (Hershko et al., 1983). The four steps of isopeptide bond formation (and the participating enzymes of the ubiquitin–protein ligase system) are ubiquitin carboxy-terminal adenylation (enzyme E1), thiolester bond formation (E1), transesterification (E1 and E2), and ubiquitin–protein conjugate formation (E2 and E3) (Figure 1; see also Hershko and Ciechanover, 1982; Haas and Rose, 1982; Ciechanover et al., 1984a). At least some of the isopeptide bonds between ubiquitin and acceptor proteins can be reversed, with regeneration of both moieties, by the action of an isopeptidase, a distinct and abundant enzyme (Andersen et al., 1981; Matsui et al., 1982; see also Rose and Warms, 1983).

Independent experimental approaches have suggested that ubiquitin-mediated, nonlysosomal proteolysis occurs not only in terminally differentiating, anucleate reticulocytes but also in a variety of other mammalian cell types (Hershko et al., 1982; Chin et al., 1982). However, ubiquitin-dependent proteolysis in extracts from nucleated eucaryotic cells has not been reported. Achieving a detailed understanding of the design and functional significance of the ubiquitin system would require some means to perturb the ubiquitin system specifically in vivo.

Here we demonstrate the thermolability of ubiquitin-activating enzyme (E1) purified from ts85 cells. A cell cycle mutant, ts85 is a temperature-sensitive derivative of FM3A, a cell line established from a spontaneous mouse mammary carcinoma (Mita et al., 1980). ts85 has a complex but well defined phenotype: shift-up of ts85 cultures to a nonpermissive temperature rapidly and completely inhibits the generation of new mitotic cells (Mita et al., 1980; Matsumoto et al., 1980), and the initially unsynchronized ts85 cultures evolve a stable distribution of cell cycle positions composed of early G2 and (fewer) late S phase cells (Mita et al., 1980; Yasuda et al., 1981). The rate of histone H1 phosphorylation in vivo was measured in synchronized G2 and G1/S ts85 cultures, and in both instances ts85 showed a temperature-dependent defect (Matsumoto et al., 1980; Yasuda et al., 1981). However, both cytoplasmic and nuclear protein kinase activities from ts85 cells, tested with histone H1 as a substrate, behaved as wild type in thermoinactivation experiments in vitro (Yasuda et al., 1981), suggesting that the reduction in H1 phosphorylation is secondary to some other defect.

Remarkably, a temperature-sensitive modification of yet another histone (H2A) was identified. The ubiquitin–H2A semihistone, uH2A, disappeared from the ts85 chromatin

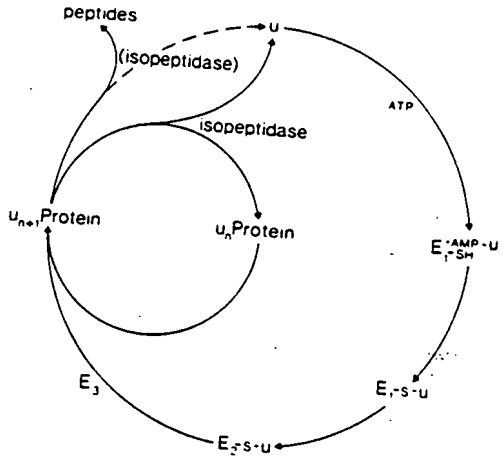

Figure 1 Proposed Pathways of the Ubiquitin System

A scheme modified from Hershko and Ciechanover (1982) and Hershko (1983). Ubiquitin (u) conjugated through high-energy bonds (~) is represented to the right of the acceptor molecule, and for low-energy conjugates ubiquitin is at the left, followed if necessary by a subscript ($n = 0, 1, 2, \ldots$) denoting the multiplicity of ubiquitin moieties covalently bound to the acceptor. The steps leading to the formation of u_{n+1}-protein conjugates are described in the Introduction. The dotted line on the left signifies a proposed but undocumented role for isopeptidase activity. The pathway from (u_{n+1}-protein) to (peptides) is not understood in detail, but probably requires several protein factors and ATP (A. Hershko, personal communication).

at 39°C with a half-life of about 3 hr (Marunouchi et al., 1980; Matsumoto et al., 1983). A temperature-resistant growth revertant, ts85R-MN3, was phenotypically wild type for both H1 phosphorylation and H2A ubiquitination, strongly suggesting that the apparently single mutation responsible for arresting the cell cycle is also responsible for the defects in histone modifications (Yasuda et al., 1981).

We demonstrate below that the primary lesion in the cell cycle mutant ts85 is in its ubiquitin–protein ligase system. In the accompanying paper (Ciechanover et al., 1984b), we show that ts85 cells, unlike the parental FM3A cells, fail to degrade otherwise short-lived intracellular proteins at the nonpermissive temperature—a previously unknown aspect of the ts85 phenotype. Taken together, our in vitro and in vivo findings with ts85 demonstrate that the degradation of the bulk of short-lived intracellular proteins in this higher eucaryotic cell proceeds through a ubiquitin-dependent pathway. The ubiquitin–protein ligase system is shown here to be essential for both cell cycle progression and cell viability. We discuss possible roles of the ubiquitin system in DNA transactions, the heat shock response, and the cell cycle.

Results

Ubiquitin-Conjugating Activity in Crude Extracts from ts85 Cells Is Specifically Thermolabile

We have used an in vitro assay for ubiquitin–protein conjugation (Ciechanover et al., 1980) based upon direct visualization, by SDS gel electrophoresis and ^{125}I-autoradiography, of in vitro-synthesized conjugates between

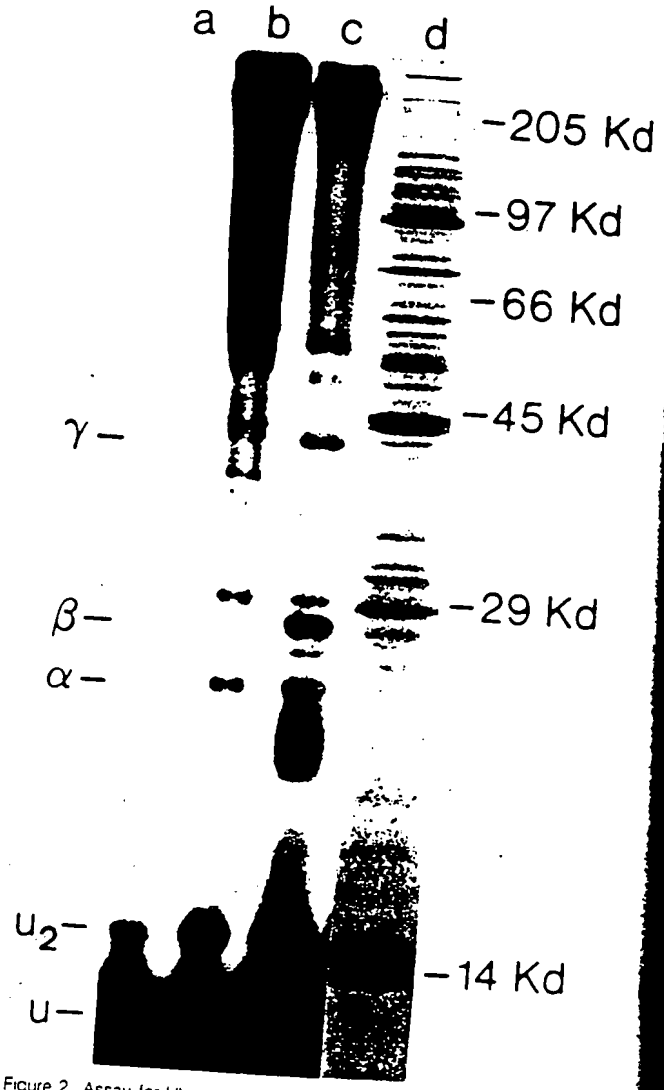

Figure 2 Assay for Ubiquitin–Protein Conjugation in Crude Extracts from ts85 Cells

ts85 cells (1×10^7) were lysed by pipetting in 100 μl of 0.25% Triton X-100, 1 mM DTT, 10 mM Tris·HCl (pH 7.6). The lysate was centrifuged at 20,000 × g for 20 min, and the supernatant was used as a source of ubiquitin-conjugating activity. Crude extract (10 μl) was assayed in a final volume of 30 μl by adding 0.65 μg (5.5×10^5 cpm) of ^{125}I-ubiquitin, with (lane c) or without (lane b) 80 μg of denatured lysozyme to the assay buffer (see Experimental Procedures). Lane d shows a Coomassie-stained protein pattern corresponding to lane c. Lane a is from a mock assay (extract omitted). Assays were at 30°C for 5 min. Samples were analyzed on discontinuous 12.5% polyacrylamide–SDS gels. α, β, and γ denote bands of lysozyme containing increasing numbers of covalently linked ^{125}I-ubiquitin moieties per lysozyme molecule. u: ubiquitin. u_2: (presumptive) ubiquitin–ubiquitin conjugate. (We note that iodinated ubiquitin may be a good ubiquitin acceptor [unpublished data]. A band seen by Chin et al. [1982] upon microinjection of ^{125}I-ubiquitin into HeLa cells could correspond to our u_2 band).

added ^{125}I-labeled ubiquitin and unlabeled acceptor proteins. In crude extracts a number of endogenous proteins serve as ubiquitin acceptors (Figure 2b), and although the Coomassie-stained electrophoretic patterns of proteins in such extracts contain a number of discrete bands (Figure 2, lane d), the electrophoretic profile of endogenous high

lecular weight ^{125}I-ubiquitin–protein conjugates is characteristically smeared as a result of size heterogeneity and apparently also SDS-resistant aggregation (Figure 2, lanes b–d, and unpublished data). When this system is supplemented with denatured lysozyme, a favorable substrate for ubiquitination, the abundantly formed ^{125}I-ubiquitin–lysozyme conjugates produce discrete electrophoretic bands (Figure 2, lane c), suitable for rapid and accurate quantitation.

Extracts from ts85 and FM3A cells cultured at a permissive temperature (32°C) have comparable conjugating activity (Figures 3A, lane a, and 3C, lane a), and when extracts are prepared from cells 10 hr after shift-up to 39°C, no change is seen in the activity of FM3A extracts

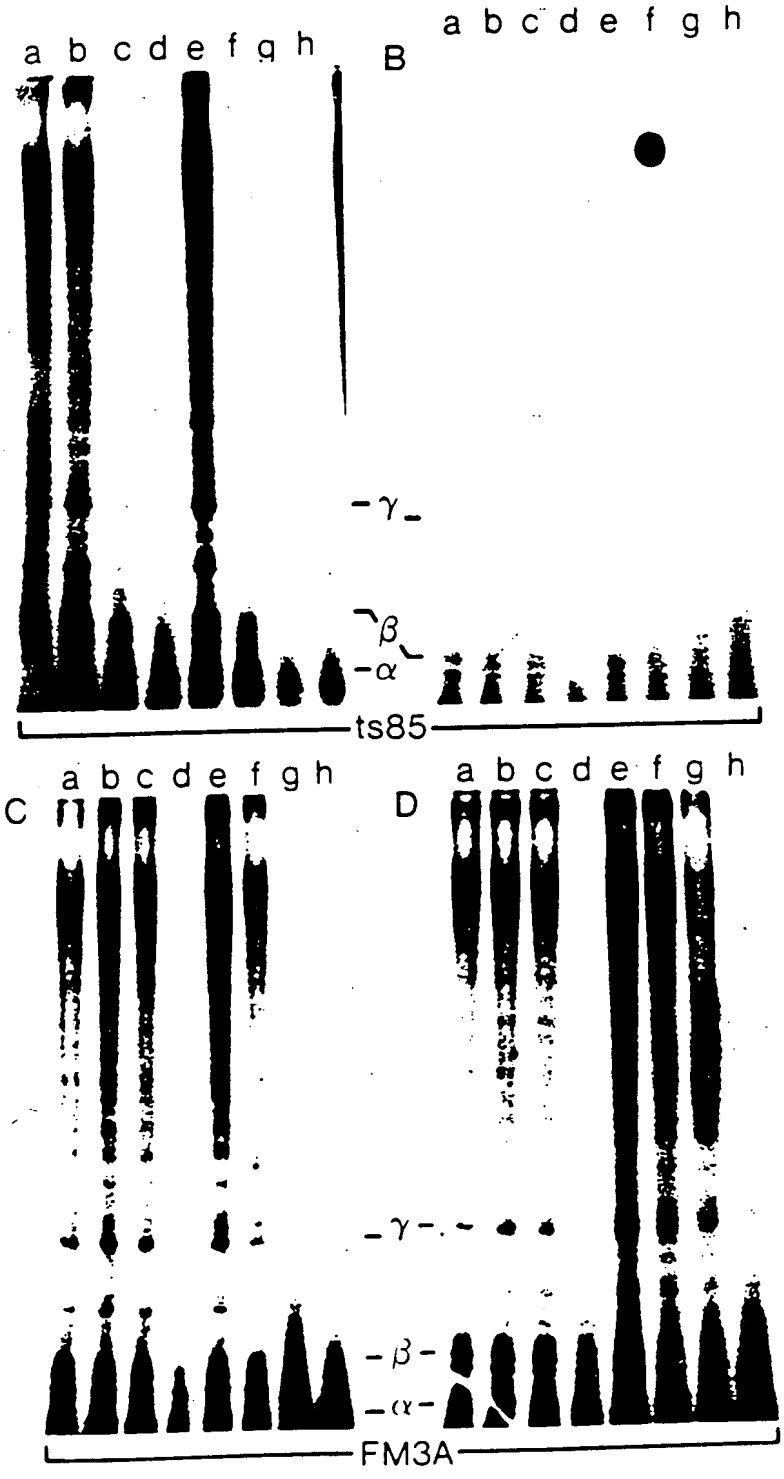

Figure 3. In Vitro Ubiquitin–Protein Conjugation in Crude Extracts from FM3A and ts85 Cells

Extracts were prepared (as in Figure 2) from parallel cultures at either 32°C (A,C) or 39°C (10 hr after shift-up, B,D). Crude extracts were assayed in a final volume of 40 μl at 4 mg/ml (protein of the extract) by adding 0.65 μg (5.5×10^5 cpm) of ^{125}I-ubiquitin and 80 μg of denatured lysozyme to the assay buffer (see Experimental Procedures). Assays were at 30°C (lane a), 35°C (lane b), 40°C (lane c), and 45°C (lane d), following preincubations of 5 min at the assay temperature. The remaining assays were at 30°C after preincubations of 2 hr at 30°C (lane e), 35°C (lane f), 40°C (lane g), and 45°C (lane h). The ts85-specific in vitro heat inactivation of ubiquitin–protein conjugation can be seen using either of the two assays (lanes a–d or e–h). 2.3×10^5 cpm from these samples were analyzed on discontinuous 12.5% polyacrylamide–SDS gels. Autoradiographic exposure was for 3 days. The low molecular weight region of the gel, containing the unconjugated ^{125}I-ubiquitin, is not shown. α, β, and γ denote bands of lysozyme containing increasing numbers of covalently linked ^{125}I-ubiquitin moieties per lysozyme molecule.

(Figure 3D, lane a). In striking contrast, extracts from ts85 cells cultured at 39°C have greatly reduced ubiquitin-conjugating activity with both endogenous and exogenous protein substrates (Figure 3B, lane a). Moreover, in extracts from cells grown at the permissive temperature, heat inactivation in vitro was specific for the ts85 ubiquitin-conjugating activity (Figure 3A).

The ts85 extracts from 39°C cultures have a small residual ubiquitin-conjugating activity (Figure 3B, lane a). The in vitro heat lability of this residual activity (Figure 3B) is comparable to that of the wild-type ubiquitin-conjugating activity in FM3A extracts (Figures 3C, 3D, and data not shown). This suggests that a heat-induced conformation of the putative heat-sensitive ubiquitin-conjugating enzyme may actually retain detectable activity, or perhaps that the pool of this enzyme in vivo is heterogeneous in thermolability, so that specifically surviving subpopulations of the enzyme would have reduced thermolability in vitro.

In summary, the results shown in Figure 3 suggest a heat-induced inactivation in vivo of a component of the ubiquitin–protein ligase system in ts85 but not in FM3A cells, and furthermore, that this phenomenon is closely simulated in cell lysates. Partially purified ubiquitin–protein ligase preparations (fraction II: see Experimental Procedures), free of ubiquitin and ubiquitin–protein conjugates, behaved in thermoinactivation experiments (data not shown) identically with the crude preparations in Figure 3, indicating that the ts85 ubiquitin conjugation defect is not due to, or dependent on, the presence of ubiquitin from ts85 cells.

ts85R-MN3 is a characterized growth revertant of ts85 (Yasuda et al., 1981), derived from ts85 by chemical mutagenesis and selection for the wild-type phenotype of cell cycle progression. The kinetics of heat inactivation of fraction II (see Experimental Procedures) prepared from ts85R-MN3 cells was indistinguishable from that of fraction II from wild-type FM3A cells (data not shown). The in vitro thermolability of the ts85 ubiquitin-conjugating activity is therefore specifically associated with the phenotype of cell cycle arrest of ts85 cells at the nonpermissive temperature.

Constraints on putative mechanisms of inactivation can be inferred from the behavior of mixed (in this case, ts85 and FM3A) extracts. From the additivity of ubiquitin-conjugating activity observed when mixed extracts are inactivated by heat (Figure 4A), we infer that catalytic enzyme modification (e.g. phosphorylation, dephosphorylation, proteolysis) by an exchangeable activity peculiar to ts85 extracts is not likely to be a rate-determining step of heat inactivation. If such an inhibitory activity did exist in the ts85 extract, one would expect the activity of the mixed extract to resemble ts85 in its temperature sensitivity. Results similar to those in Figure 4A were obtained in an

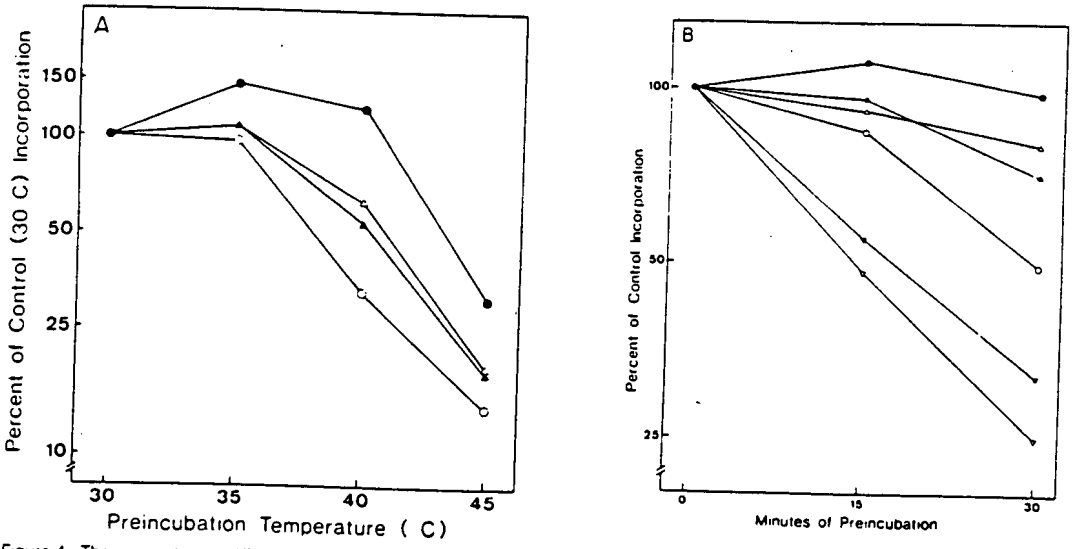

Figure 4. Thermoinactivation Kinetics of Ubiquitin–Protein Conjugation in Mixed Extracts

(A) Extracts, prepared from exponentially growing cells after depletion of their ATP pools at 30°C (see Experimental Procedures), were fractionated on DEAE-cellulose to yield fraction II (see Experimental Procedures). Fraction II from ts85 and fraction II from FM3A cells were incubated either separately at 4 mg/ml (protein of the extract) in the presence of added ovalbumin at 4 mg/ml, or mixed 1:1 and assayed (with ^{125}I-ubiquitin and lysozyme as described for Figure 3) at 8 mg/ml total protein. Assays were carried out at 30°C after preincubations of 2 hr at different temperatures, as indicated. ATP (0.5 mM) was present during preincubations and assays. The samples were analyzed on an SDS gel, band γ (see Figure 3) was excised from specific lanes of the dried gel, and the ^{125}I radioactivity was determined using a gamma counter. (-●-) FM3A extract. (-○-) ts85 extract. (-Δ-) mixed extract of ts85 plus FM3A. (-▲-) mixed extract, calculated additive curve. The calculated curve was derived by adding the cpm values for the appropriate bands (prior to normalization), then normalizing. Band γ was used for quantitation because it is in an area of the gel having low nonspecific ^{125}I background.

(B) Bands X and uH2B were excised from specific lanes of the dried gels shown in Figure 6 and the ^{125}I radioactivity was determined using a gamma counter. Band uH2B: (-○-) ts85, Figure 6A, lanes a–d; (-●-) FM3A, Figure 6B, lanes a–e; (-Δ-) mixed extract of ts85 plus FM3A, gel not shown; (-▲-) mixed extract of ts85 plus FM3A, calculated additive curve. Band X. (-▽-) ts85, Figure 6A, lanes a–c); (-▼-) mixed extract. Band X in the FM3A sample was not sufficiently radioactive to quantify. The slight apparent stabilization of band X in the mixed extracts may be due to the small component of the total cpm in band X contributed by wild-type band X. The mixing experiment and the corresponding calculations were carried out essentially as in Figure 4A.

analogous mixing experiment carried out with affinity-purified ubiquitin-conjugating enzymes from ts85 and FM3A cells (Figure 4B).

Both Ubiquitin Synthesis and Ubiquitin Conjugation to Histone H2A Are Inhibited in ts85 Cells at Nonpermissive Temperature

To address the significance of our in vitro results we attempted to show directly a loss of ubiquitin-conjugating activity in ts85 cells in vivo. After a sufficiently brief pulse labeling with ^{35}S-methionine, a decrease in fluorographic intensity of the uH2A electrophoretic band (relative to that of free ubiquitin) would be seen only if the rate of in vivo ubiquitin conjugation to H2A were reduced. The synthesis of both free ubiquitin and uH2A were monitored, since the bulk of ^{35}S-methionine pulse label in uH2A is within its ubiquitin moiety (see Seale, 1981, and the legend to Figure 5).

ts85 cells were labeled for 10 min with ^{35}S-methionine at 30.5°C and at 39°C, the latter either 70 min or 140 min after the shift-up. Whole-cell acid extracts were subjected to electrophoresis in a composite gel system (see Experimental Procedures) developed to monitor the in vivo synthesis of uH2A and free ubiquitin (Figure 5). The gel was loaded with equal amounts of TCA-insoluble ^{35}S-methionine counts. One unanticipated finding from this experiment was that synthesis of free ubiquitin was significantly and specifically inhibited in ts85 cells at the nonpermissive temperature (Figure 5B). Although a transient effect of apparently the same nature was observed with both parental FM3A cells (data not shown) and revertant ts85R-MN3 cells (Figure 5B, lane e), in ts85 cells ubiquitin synthesis remained inhibited for the duration of treatment at the nonpermissive temperature (at least 13 hr, the longest period monitored: Figure 5B, and data not shown). We suggest these results are due to a specific regulation of ubiquitin synthesis. One possibility is that ubiquitin synthesis is controlled via negative feedback according to the levels of free ubiquitin. This would explain the inhibition of ubiquitin synthesis in ts85 cells at the nonpermissive temperature as the result of an isopeptidase-mediated release of free ubiquitin from ubiquitin–protein conjugates.

The composite polyacrylamide gel, in which free ubiquitin is resolved in one dimension (Figure 5B), served also as the first of two electrophoretic dimensions required to resolve uH2A in the same sample of whole-cell acid extract. Only 70 min after shifting ts85 cells to 39°C, a strong (at least 75%) reduction in the fluorographic intensity of the band of the newly synthesized uH2A was observed (Figure 5A). This result can be compared to an approximately 25% reduction in the steady-state (total) uH2A levels in ts85 after 1 hr at 39°C (Marunouchi et al., 1980). As mentioned above, the fluorographic intensity of the band of newly synthesized free ubiquitin at 39°C is also reduced in ts85, but to a much smaller extent (Figure 5).

A different two-dimensional electrophoretic system (see Experimental Procedures) was used to confirm that the residual intensity of the uH2A band in Figure 5A is due at least mostly to uH2A, rather than an unrelated minor comigrating protein (data not shown). We interpret this low

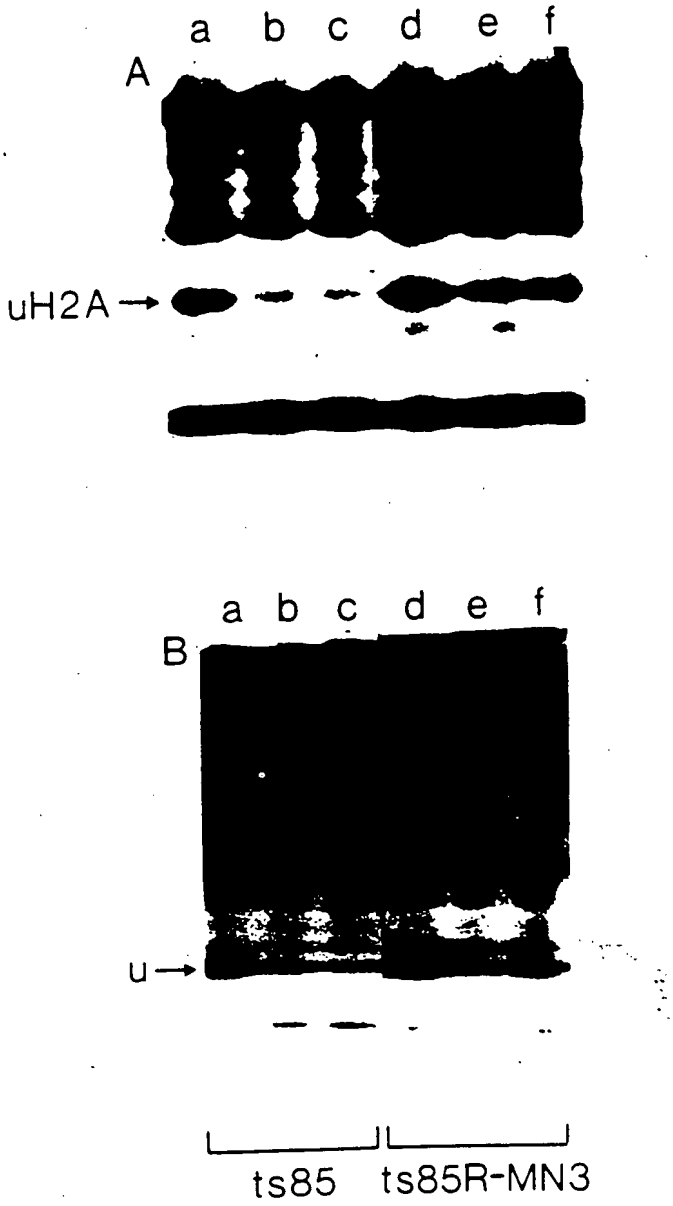

Figure 5. Both Ubiquitin Synthesis and Ubiquitin Conjugation to Histone H2A Are Inhibited in ts85 Cells at the Nonpermissive Temperature: Analysis by In Vivo Pulse-Labeling

ts85 cells (lanes a–c) and ts85R-MN3 cells (lanes d–f) were pulse-labeled with ^{35}S-methionine for 1 min (see Experimental Procedures), either at 30°C (lanes a,d) or at 40°C, in the latter case either 70 min (lanes b,e) or 140 min (lanes c,f) after shift-up. Whole-cell acid extracts (1.5×10^5 TCA-insoluble cpm per lane; see Experimental Procedures) were subjected to electrophoresis in composite gels (see Experimental Procedures) and fluorography to monitor the in vivo synthesis of uH2A (A) and ubiquitin (B). A separate experiment showed that the synthesis of H2A.2 (the only subtype of mouse H2A containing methionine; West and Bonner, 1980) is not temperature sensitive in relation to the other core histones (data not shown). See Experimental Procedures for the electrophoretic techniques and other details. uH2A: ubiquitin–H2A semihistone. u, ubiquitin.

but detectable rate of uH2A formation by ts85 cells at the nonpermissive temperature (Figure 5A) as a result of leakiness in the temperature sensitivity of the ubiquitin-conjugating system in vivo. This interpretation is consistent with our in vitro results (Figure 3B).

Most importantly, the in vivo experiment of Figure 5 shows that the previously observed disappearance of uH2A from ts85 cells at the nonpermissive temperature (Marunouchi et al., 1980; Matsumoto et al., 1983) is due to reduction of uH2A synthesis, rather than enhanced degradation. The rapidity of the effect of temperature shift-up on ubiquitin–protein conjugation strongly suggests that the numerous other temperature-induced defects in ts85 cells (see Introduction) are pleiotropic effects of deficient ubiquitin–protein conjugation.

Thermolability of Affinity-Purified Ubiquitin-Conjugating Activity from ts85 Cells

Affinity chromatography on ubiquitin–Sepharose (Hershko et al., 1983) was used to copurify the three enzymes (E1–E3) required for ubiquitin–protein conjugation. This substantially purified system was subjected to heat inactivation, and subsequent ubiquitin-conjugation assays (in the presence of added histone H2B as a ubiquitin acceptor; see Experimental Procedures) showed that the specific thermolability of the ubiquitin–protein ligase system from ts85 cells had been retained (see Figure 6).

In addition to this confirmatory result, Figure 6 shows that, unlike the wild-type ubiquitin–protein ligase system, the ts85 system strongly conjugates ubiquitin to a specific endogenous substrate (band X; Figure 6A, lane a). Since the substrate specificity of the ubiquitin–protein ligase system includes a high selectivity for structurally aberrant proteins (Hershko et al., 1982; Chin et al., 1982), it seemed possible that the ^{125}I-ubiquitin was covalently bound to the mutant enzyme we sought to identify. In the ts85 samples, the intensities of both uH2B and X bands decreased with time spent at 40°C (Figures 4B and 6). Unexpectedly, however, the decay of band X was not reversed by the presence of an active (wild-type) ubiquitin–protein ligase system (Figure 4B). Apparently any putative heat-induced structural damage to the unlabeled precursor of band X reduced, rather than magnified, the probability of its ubiquitination. Upon prolonged exposure, band X could be visualized in the FM3A samples as well (Figure 6D). The staining intensity of an unlabeled protein band immediately beneath the band X, identified below as the nonubiquitin component of band X (Figure 6E), was similar in ts85 and FM3A samples (data not shown). A faint high molecular weight band (band Y), well separated from band X, appeared in the ts85 samples only upon a 40°C preincubation (Figure 6C). The same band appeared in the FM3A sample after an additional 45 min at 40°C (Figure 6D).

Quantitative analysis (Figure 4B) showed that in the ts85 sample the band X intensity decreased more rapidly than that of uH2B. In addition, the decay of band X, unlike that of band uH2B, was exponential and extrapolated to the control, or time zero, value. These characteristics suggested that the decrease in band X intensity quantitatively traced the primary inactivation event in the ts85 sample. Strong independent support for this interpretation is found in the mixing experiment, in which the quantitative features of band X decay are conserved (Figure 4B). The results obtained using mixed (ts85 plus FM3A) extracts (Figure

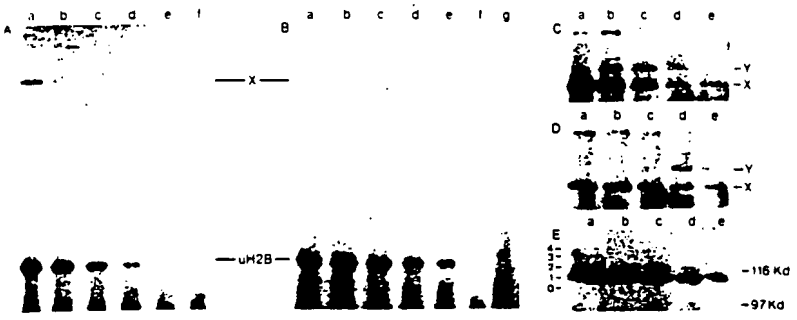

Figure 6. Heat Inactivation of Affinity-Purified Ubiquitin–Protein Ligase Systems from ts85 and FM3A Cells

Ubiquitin–protein ligase activity was purified from fraction II by ubiquitin-affinity chromatography (elution with 2 mM DTT, 50 mM Tris·HCl, pH 9.0; see Experimental Procedures). Purified ubiquitin–protein ligase samples from ts85 cells (A,C) and from FM3A cells (B,D) were preincubated at 40°C prior to assay with ^{125}I-ubiquitin at 30°C for 5 min. The time points of preincubation at 40°C are (in minutes) 0 (lane a), 15 (b), 30 (c), 60 (d), 90 (e), and 120 (f). Ovalbumin 5 mg/ml), pyrophosphatase (10 units/ml), and ATP (2 mM) were present throughout the experiment. Ubiquitin–protein ligase sample was omitted from the assay in B, lane g. The ligase samples were assayed at 60 μg/ml, with purified histone H2B at 2 mg/ml as the ^{125}I-ubiquitin acceptor and ^{125}I-ubiquitin at 8 μg/ml. 2 × 10^5 cpm were loaded onto each lane of an 18% discontinuous polyacrylamide–SDS gel. A and B are 24 hr autoradiographic exposures, and C and D (upper regions of the gels shown in A and B, respectively), are 10 day exposures. uH2B denotes a band dependent on the addition of exogenous substrate, histone H2B purified from calf thymus. This band is tentatively identified as monoubiquitinated H2B (uH2B) by the criterion of electrophoretic mobility (data not shown). We have not determined whether the ubiquitination site(s) of in vitro-synthesized uH2B is identical with that of in vivo-synthesized uH2B (West and Bonner, 1980). Neither form is known to be homogeneous in this regard. X and Y denote high molecular weight ubiquitin–protein conjugates identified in E (see text and Table 1). E shows the high molecular weight region of a discontinuous 7.5% polyacrylamide–SDS gel loaded with samples described elsewhere, as indicated: lane a (A, lane c); lane b (Figure 6A, lane a); lane c (Figure 6B, lane a); lane d (Figure 8B, lane j); and lane e (Figure 8B, lane f). The time of autoradiographic exposure was separately chosen for each lane in E, to optimize the visualization of discrete bands. The bands designated 0, 1, 2, 3, and 4 correspond to E1 enzyme conjugated to the indicated number of ubiquitin molecules. The location of nonubiquitinated E1 (band 0) was found by superimposing the autoradiogram and the stained gel (data not shown). Band 4 is faint in the autoradiogram and could not be reproduced satisfactorily.

B) indicate that the rate of ubiquitination of the precursor ... band X is independent of the rate of ubiquitination of histone H2B in the same sample.

Selected samples were analyzed on a more dilute (7.5%) polyacrylamide-SDS gel for molecular weight estimation and better resolution in the high molecular weight region. The apparent molecular weight of band X (~113 kd) is consistent with its assignment as monoubiquitinated ubiquitin-activating enzyme (uE1). No such modification of E1 had been reported, but the well characterized rabbit reticulocyte E1 (Haas and Rose, 1982) did form uE1 when ...ed (Figure 6E, lane e). The ubiquitinated E1 species observed in Figure 6 are distinct from the previously characterized thiolester between E1 and ubiquitin (E1-S~u; Ciechanover et al., 1982; Haas et al., 1982; see also Figure 1), since the former are resistant to boiling in 0.65 M 2-mercaptoethanol prior to electrophoresis, a treatment known to cleave the E1-S~u thiolester efficiently (Ciechanover et al., 1982). The possibility that band X is simply the residual of an incomplete cleavage of the thiolester bond ...cluded by the fact that band X is resolved into a ...blet (consisting of uE1 and u_2E1; Figure 6E, lane d, and data not shown) on a 7.5% polyacrylamide-SDS gel; residual E1-S~u would be expected to be a unique species and therefore to migrate as a singlet instead of a doublet. In the 7.5% polyacrylamide-SDS gel, the relative mobility of band Y was shifted toward this new doublet, resulting in an array of bands that was clearly periodic (Figure 6E, lane a, Table 1), much as previously observed in electrophoretic patterns of multiubiquitinated proteins (Hershko ...1980).

...m the exponential decay of band X in thermoinactivation experiments (Figure 4B) we infer that uE1 and u_2E1 (which together comprise the band X; Figure 6, Table 1, and data not shown) form simply in proportion to the amount of active E1 enzyme from ts85 cells. In contrast, generation of detectable u_3E1 and u_4E1 (band Y in Figure 6) apparently requires a metastable E1 species (stable at 4°C and for at least 5–10 min at 30°C) formed only upon heat treatment of the ubiquitin–protein ligase system.

...molability of Ubiquitin-Activating Enzyme from ts85 Cells

Seeking to show directly that E1 from ts85 cells was temperature sensitive, we assayed the formation of ubiquitin-E1 thiolester E1-S~u (an intermediate in the transfer of activated ubiquitin to acceptor proteins; see Figure 1 and Ciechanover et al., 1982). If the ubiquitinating enzymes E2 and E3 were separated from E1, temperature-sensitive formation of E1-S~u by the E1 enzyme from ts85 cells ... be interpretable directly in terms of E1 activity and ... not be ascribed to altered function in a downstream enzyme. E1 was purified to more than 90% homogeneity (Figure 7) by covalent ubiquitin-affinity chromatography, using pyrophosphate and AMP to drive the back-reaction of ATP synthesis that elutes E1 from the ubiquitin column (Ciechanover et al., 1982; see also Figure 1). We found no difference between the electrophoretic mobilities of E1 enzymes from ts85 and FM3A cells, but a small reproducible difference, corresponding to about 2 kd, between ts85 and rabbit reticulocyte E1 enzymes, probably resulting from a species difference (Figure 7, lanes a and b). The rabbit and mouse forms of (presumptive) uE1 also showed this small difference in electrophoretic mobility (Figure 6E, lanes d and e; compare to Figure 7, lanes b and a). Since the protein stain (Figure 7) definitely visualizes E1 enzyme

Table 1. Ubiquitinated Derivatives of Ubiquitin-Activating Enzyme (E1)

Electrophoretic Band Designation	Apparent Molecular Weight (kd)	Putative Identity
0	105	E1
1	113	uE1
2	118	u_2E1
3	124	u_3E1
4	120	u_4E1

Each electrophoretic band is designated by a number corresponding to the putative multiplicity of ubiquitin moieties (n) in the corresponding u_nE1 derivative. Rabbit reticulocyte E1 formed uE1 but not u_2E1 (Figure 6E, lane e). See Figure 6 and the main text for details.

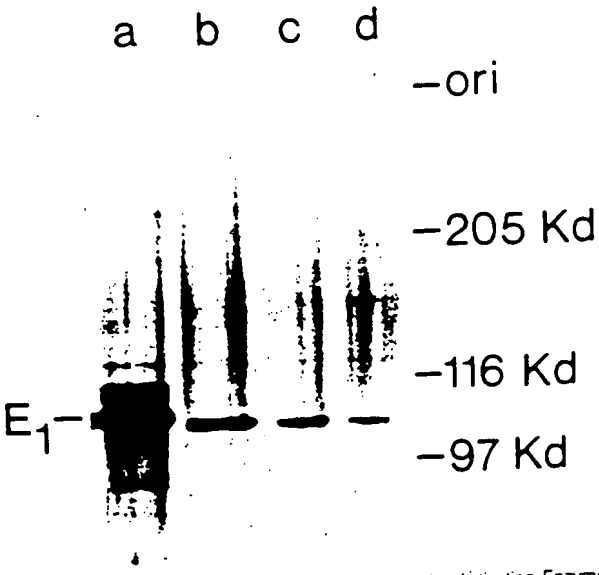

Figure 7. Electrophoretic Pattern of Purified Ubiquitin-Activating Enzyme (E1) from ts85 Cells: Comparison with Homologous Enzyme from Rabbit Reticulocytes

Fraction II was further fractionated by covalent affinity chromatography on a ubiquitin–Sepharose column (elution with 2 mM AMP, 100 μM Na-pyrophosphate, 50 mM Tris-HCl, pH 7.2; see Experimental Procedures). The affinity column was loaded with 44 mg of extract (fraction II) from ts85 cells; lane b was loaded with 1/8 of the column eluate; lane c, 1/25; lane d, 1/80. Lane a was loaded with 1/8 of the affinity column eluate from 28 mg of rabbit reticulocyte extract (fraction II). E1 was similarly purified from 12 mg of fraction II from FM3A cells (not shown). The origin of the 7.5% polyacrylamide–SDS separating gel is indicated. Proteins were visualized by silver staining (see Experimental Procedures). The estimated purity (compare lanes b and d) of affinity-purified ubiquitin-activating enzyme (E1) from ts85 cells is more than 90%.

(Ciechanover et al., 1982; Haas et al., 1982), the corresponding mobility difference between the autoradiographic bands (Figure 6E, lanes d and e, and data not shown) clearly identifies their unlabeled precursor as E1 enzyme.

Figure 8 shows equilibrium levels of E1-S~u formed upon 30°C incubation of purified E1 from ts85 and FM3A cells in the presence of ^{125}I-ubiquitin and ATP (Ciechanover et al., 1982; Haas et al., 1982; see also Figure 1). A 40°C preincubation rapidly and specifically inactivates E1 enzyme from ts85 (Figure 8A, lanes b–e). Thus heat inactivation very likely slows or prevents the formation of E1-S~u; however, destabilization of E1-S~u from ts85 cells may also contribute to its disappearance upon heat treatment, as observed by equilibrium assay (Figure 8). When directly assayed as in Figure 8, the wild-type E1 enzyme is quite stable at 40°C, suggesting that the slow inactivation of the wild-type system observed in Figure 6 might be due in part to heat inactivation of another enzyme of the ubiquitin–protein ligase system.

In the experiment shown in Figure 8A, the control (30°C) ts85 E1 activity was much lower than the corresponding wild-type (FM3A) E1 activity. Since E1 is known to form a homodimer (Ciechanover et al., 1982), a concentration-dependent monomer–dimer equilibrium could exist. As-

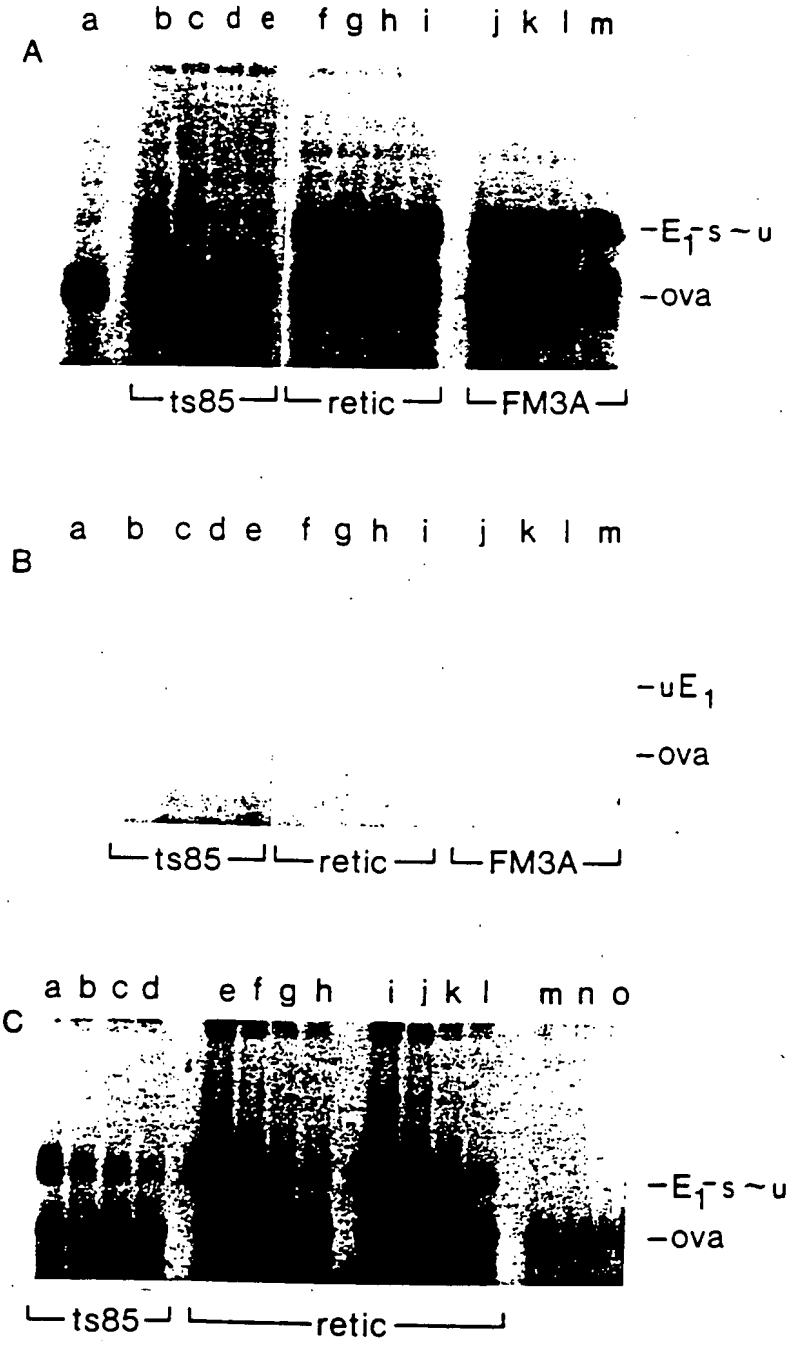

Figure 8. Heat Inactivation of Purified Ubiquitin-Activating Enzymes (E1) from ts85, FM3A, and Rabbit Reticulocytes

E1 enzymes were purified by ubiquitin-affinity chromatography (see legend to Figure 7 and Experimental Procedures). Preincubation at 40°C was in 10% glycerol, 0.2 mM DTT, 5 mg/ml ovalbumin, 2 mM ATP, 5 mM $MgCl_2$, 50 mM Tris·HCl (pH 7.2). In (A) and (B) the times of preincubation at 40°C were 0 (lanes b,f,j), 20 (lanes c,g,k), 40 (lanes d,h,l), or 80 (lanes e,i,m) min. Lanes a in A, B, and C are mock assays in which ubiquitin-activating enzyme was omitted. In C, preincubation at 40°C was for 0 (lanes a,e–h,m), 10 (lane b), 20 (lane c), and 40 (lanes d,i–l) min. The reticulocyte enzyme was preincubated and assayed without dilution (lanes e,i), and after dilutions of 3-fold (lanes f,j), 9-fold (lanes g,k), and 27-fold (lanes h,l). In C, lane n is a control identical with lane e, except that the SDS sample buffer was added before the ^{125}I-ubiquitin; lane o is the corresponding mock assay (enzyme omitted). The enzyme assay (50 μl) was initiated after preincubation at 40°C by the addition of ^{125}I-ubiquitin (0.45 μg), at 30°C, and pyrophosphatase (0.62 unit). After 10 min at 30°C, 20 μl samples were mixed with equal volumes of SDS sample buffers, either with (B) or without (A and C) 2-mercaptoethanol (0.65 M final concentration). The 2-mercaptoethanol-containing samples were boiled for 3 min prior to electrophoresis to cleave the thiolester bond between ubiquitin and ubiquitin-activating enzyme (E1); electrophoresis was in 7.5% discontinuous polyacrylamide–SDS gels (15 cm long, 0.8 mm thick) at 4°C and 50 ma, with additional cooling by a fan. Before drying, the gels were soaked in 5% glycerol for 10 min. Each lane was loaded with 7×10^5 cpm. In A and B, exposure times were 12 hr for lanes a–e, and 6 hr for lanes f–m. C was exposed for 16 hr. E1-S~u denotes a monoubiquitinated derivative of E1 coupled to ubiquitin via a thiolester bond. uE1 denotes the minor 2-mercaptoethanol-resistant residual from this band, a species distinct from the thiolester derivative E1-S~u (see text); ova denotes the band of carrier ovalbumin, detected by Coomassie staining (not shown). The association of a small proportion of the free ^{125}I-ubiquitin with this band is not dependent on E1 (A, lane a) and is apparently noncovalent (B, lane a).

suming that only E1 monomers were heat sensitive, the rapid thermoinactivation of the E1 enzyme from ts85 could reflect merely a higher fractional monomer concentration in the ts85 sample. However, in a second experiment (Figure 8C, lanes a and d; compare to lanes h and l), interpretations of this type were excluded by assessing inactivation as a function of the wild-type enzyme concentration. We conclude that ubiquitin-activating enzyme (E1) from ts85 cells is thermolabile.

Discussion

Thermolability of Ubiquitin-Activating Enzyme from ts85 Cells: Possible Interpretations

We have shown by pulse-labeling in vivo that the loss of uH2A in ts85 cells at nonpermissive temperature is due to reduced ubiquitin–protein conjugation, and that the reduced conjugation rate is due ultimately to the specific thermolability of ubiquitin-activating enzyme E1 from ts85 cells. Thus the mutation controlling conditional cell cycle arrest in ts85 cells is predicted to lie within the structural gene for E1. Since the ts85 phenotype is recessive (Yasuda et al., 1981), the corresponding mutation may be rescuable by transfection with wild-type DNA. This approach could be used to clone the E1 gene and thereby ultimately to exclude the remote possibility that a constitutive defect in posttranslational modification accounts for the thermolability of the E1 enzyme from ts85 cells.

We have also characterized multiubiquitinated derivatives of E1 enzyme formed in affinity-purified in vitro systems (Figure 6 and Table 1). These derivatives are distinct from ubiquitin–E1 thiolester, and had not been previously identified. The thermolabile E1 enzyme from ts85 accepts ubiquitin at a much higher rate than the wild-type enzyme; this initially implicated E1 as the temperature-sensitive component of the ts85 ubiquitin–protein ligase system. Neither the physiological significance of the ubiquitination of E1 nor its occurrence in vivo is addressed by our experiments. One might expect that the synthesis of u_nE1 occurs via the three-component ubiquitin–protein ligase pathway (Figure 1). In this case, the unexpectedly high rate of u_nE1 synthesis could be due to the presence of a high-affinity (0.58 μM; Haas and Rose, 1982) binding site for ubiquitin on the E1 enzyme (the site of ubiquitin adenylate synthesis; see Figure 1). If the effect of 40°C preincubation on E1 (see Figures 4B and 6) were to eliminate or strongly reduce the affinity of this site for ubiquitin, the activity of the E1 enzyme and its preferred substrate status would be lost coordinately, in agreement with our observations. Although without additional ad hoc assumptions this model appears to be inconsistent with the detailed kinetics of band X decay in E1 thermoinactivation experiments (Figure 4B), neither this discrepancy nor the formation of u_nE1 in E1 preparations of more than 90% purity (Figure 6 and data not shown) suffices to preclude the above model.

An alternative model in which E1 ubiquitination requires only E1 is consistent with the high rate of u_nE1 synthesis in vitro, its specificity for active E1, and the exponential decay of band X in thermoinactivation experiments (Figure 4B). Activated ubiquitin for self-ubiquitination of E1 could be donated by either thiolester or adenylate intermediates (Figure 1). The defective E1 enzyme of ts85 may self-transfer ubiquitin with a much higher efficiency than the wild-type E1 does, either because of a structural difference in (or accessible to) its active site or as a result or a secondary effect—for example, of impaired forward reactions. For instance, the E1 from ts85 could be impaired in a postulated slow conformational change preceding the conversion of ubiquitin adenylate to ubiquitin thiolester (Figure 1, and I. Rose, personal communication).

The Ubiquitin System and the Heat Shock Response

We have observed that upon shift-up to the nonpermissive temperature, the synthesis of certain heat shock proteins is strongly induced in ts85 but not FM3A cells (D. Finley, unpublished data; see also Ciechanover et al., 1984b). This result may suggest the existence of a protein that is both an activator (presumably transcriptional) of certain heat shock genes and a preferred substrate for the ubiquitin-dependent proteolytic system. As a corollary, the rate of ubiquitin-dependent degradation of this hypothetical activator protein would be a decisive parameter regulating the induction of the heat shock response. Agents that effectively stabilize this activator against ubiquitin-dependent degradation would then act as heat shock inducers. Thus the induction of heat shock proteins would occur when the ubiquitin-dependent system were overloaded or inactivated. This is consistent with the observation by Glover (1982) that uH2A disappears upon heat shock of cultured Drosophila cells.

It has previously been shown that the synthesis of abnormal proteins, such as those containing amino acid analogs, leads to heat shock induction (Hightower and Smith, 1978; Hightower, 1980; Kelley and Schlesinger, 1978). Since denatured, or abnormal proteins, which ordinarily undergo very rapid ubiquitin-dependent degradation (see Ciechanover et al., 1984b), would be expected to accumulate in ts85 cells at the nonpermissive temperature, there emerges a possibility that the ubiquitin-dependent proteolytic pathway and the heat shock response are complementary systems designed (among other things) to prevent cellular damage that abnormal proteins could inflict. The intracellular precipitation of abnormal proteins could trap otherwise dynamic fibrous structures such as chromatin and components of the cytoskeleton. It is possible that at least some of the heat shock proteins recognize the same binding sites by which abnormal proteins recognize each other in precipitate formation, but bind monovalently to abnormal proteins so as to prevent precipitate formation. The putative capacity of heat shock proteins to provide monovalent recognition of exposed hydrophobic surfaces of proteins could play roles in aspects of

normal physiology, such as the intracellular transport of proteins destined for a specific membrane association, as in the well characterized case of the complex between the *src* protein and the heat shock protein pp90 (Courtneige and Bishop, 1982; Brugge et al., 1983). Altered properties of the heat shock response in ts85 cells (see above) may therefore prove useful in studies of the function, as well as regulation, of the heat shock response.

Possible Significance of Nuclear Ubiquitin–Histone Conjugates

The thermolability of the soluble ubiquitin-activating enzyme E1 and the inhibition of synthesis of the nuclear uH2A semihistone in ts85 cells at the nonpermissive temperature (see Results), taken together, strongly suggest that the cytoplasmic and nuclear functions of ubiquitination depend on a shared E1 enzyme. The thermolabile E1 enzyme of ts85 can thus be used to analyze both nuclear and cytoplasmic functions of ubiquitinated proteins.

The chromosomal semihistone uH2A is apparently the most abundant ubiquitinated protein species in nucleated mammalian cells (Chin et al., 1982; Atidia and Kulka, 1982). We have noted that a sequence of five amino acid residues that includes the ubiquitination site of H2A is conserved in all of the many sequenced H2As, from yeast to man, although the flanking sequences of the carboxy-terminal H2A "tail" are only partially conserved (Choe et al., 1982). As histones turn over slowly, the significance of uH2A and other ubiquitin–histone conjugates may be unrelated to proteolysis. One possibility is that in ts85 cells the specific temperature-dependent defects in chromosomal processes, such as H1 phosphorylation, DNA synthesis, and chromatin condensation (reviewed by Marunouchi et al., 1983), are due to the loss of uH2A as a specific structural component of chromatin, and not to the loss of the ubiquitin-dependent pathway of protein degradation (see Ciechanover et al., 1984b). Among the possible nonproteolytic roles of nucleosome ubiquitination are transient perturbations (unfolding) of chromatin structures, including possibly the nucleosome itself, and marking specific regions of chromosomal fibers for binding of unidentified ligands involved in chromosomal processes such as transcription or repair.

The previously reported absence of uH2A from isolated metaphase chromosomes (Matsui et al., 1979; Wu et al., 1981) has been interpreted as reflecting a causative role of the uH2A "removal" in triggering mitotic chromosome condensation. However, the predominantly dispersed nature at the nonpermissive temperature of chromatin in the ts85 cells lacking uH2A (Mita et al., 1980) strongly suggests that the loss of uH2A during G2 is not sufficient for chromosome condensation. Also, the coisolation of isopeptidase with metaphase chromosomes (Matsui et al., 1982) reduces confidence that metaphase chromosomes lack uH2A in vivo, since isopeptidase inhibitors were not used during chromosome isolation.

The previously observed preferential localization of ubiquitinated nucleosomes within transcribed chromosomal regions in Drosophila (Levinger and Varshavsky, 1982; Varshavsky et al., 1983) and the more recent finding of a highly preferential ubiquitination of the first two to three nucleosomes downstream from the transcriptional promoter of the 31 kb long mouse dihydrofolate reductase gene (J. Barsoum and A. Varshavsky, unpublished data), are both consistent with the possible nonproteolytic functions of ubiquitin–histone conjugates discussed above. These data are also consistent with the hypothesis of chromosomal locus-specific, ubiquitin-dependent proteolysis of histones as a mechanism of chromatin remodeling (Levinger and Varshavsky, 1982; Varshavsky et al., 1983). The extreme stability of differentiated cellular phenotypes may suggest an involvement of mechanistically irreversible, locus-specific modifications of chromatin structure; our working hypothesis is that the nuclear ubiquitin-dependent proteolytic system provides such a pathway. Histone displacement from the entire chromosome complement, such as occurs in spermatocyte nuclei, could also be effected by the ubiquitin-dependent proteolytic pathway, for instance, if its specificity were subject to developmental control. These hypotheses are open to test.

ts85 cells fail to degrade short-lived intracellular proteins at the nonpermissive temperature (Ciechanover et al., 1984b), demonstrating that the turnover of the bulk of short-lived intracellular proteins in this higher eucaryotic cell proceeds through a ubiquitin-dependent pathway. The protein degradation defect of ts85 cells suggests that their conditional G2 arrest may be a consequence of stabilization and accumulation of normally short-lived regulatory proteins, a class of proteins that could well include regulators of cell cycle progression (for discussion see Ciechanover et al., 1984b).

Experimental Procedures

Cell Culture

The cell lines FM3A, ts85 (Mita et al., 1980), and ts85R-MN3 (Yasuda et al., 1981) were gifts from Dr. H. Yasuda (University of California, Davis). They were grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with penicillin (50 units/ml), streptomycin (50 μg/ml), Na-pyruvate (110 mg/l), additional glucose (3.5 g/l), and 10% calf serum. Cells were grown in suspension using Falcon tissue culture flasks at 30.5°C in a mixture of 5% CO_2 and 95% air at 98% relative humidity.

Preparation of Fraction II

Exponential cultures were washed twice by low-speed centrifugation at room temperature in Hank's balanced salt solution supplemented with 20 mM Na-HEPES (pH 7.5), then resuspended to 2.5×10^7 cells per milliliter and incubated at 30°C for 4 hr in the same solution supplemented with 20 mM 2-deoxy-D-glucose and 0.2 mM 2,4-dinitrophenol (an ATP-depleting treatment; Hershko et al., 1978). Thereafter cells were pelleted, washed at 4°C with Hank's solution by low-speed centrifugation, and lysed at 2×10^8 cells per milliliter in 0.25 mM dithiothreitol (DTT), 10 mM Na-HEPES (pH 7.5), using a Dounce homogenizer. Lysates were centrifuged for 1 hr at 100,000 × g at 4°C, and supernatants were fractionated by step elution on DEAE-cellulose as previously described (Ciechanover et al., 1978). Proteins in the 0.02 → 0.5 M KCl eluate (fraction II) were precipitated by adding ammonium sulfate to 90% saturation; the pellet was resuspended in 1 mM DTT, 20 mM Tris·HCl (pH 7.5), and dialyzed against the same buffer for 2 days at 4°C. In some experiments sample concentration and

...ter exchange were accomplished by ultrafiltration in CF-25 Centriflo membrane cones (Amicon). Before freezing the samples, glycerol was added to 10%, and for storage over a month at −70°C, ATP was added to 0.5 mM. Protein concentration was determined by protein assay (Bio-Rad), using bovine IgG as a standard.

Ubiquitin-Affinity Chromatography

Ubiquitin was purified from human erythrocytes as previously described (Ciechanover et al., 1982). Ubiquitin was coupled to activated CH-Sepharose 4B (Sigma) as described previously (Ciechanover et al., 1982), except that the concentration of Sepharose-bound ubiquitin was approximately 20 mg ... milliliter of swollen gel. Column operations, storage, and washing ... were as previously described (Ciechanover et al., 1982; Hershko ... 1983). Extracts (fraction II; see above) were applied to the column in 5 mM $MgCl_2$, 0.2 mM DTT, 2 mM ATP, 50 mM Tris-HCl (pH 7.2). Elution protocols are described in the appropriate figure legends.

Ubiquitin–Protein Conjugation Assays

Ubiquitin–protein conjugation assays were performed in 10 mM creatine phosphate, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM ATP, 100 μg/ml creatine phosphokinase, 50 mM Tris-HCl (pH 7.5), unless stated otherwise. All assays were initiated by adding a solution containing purified ^{125}I-ubiquitin (labeled with ^{125}I using the chloramine T method; Ciechanover et al., 1980), ... an exogenous protein substrate (either denatured lysozyme or histone ... after a 5 min preincubation at the assay temperature. Variations of ... protocol are described in the figure legends. Assays were terminated by the addition of a half volume of the 3× SDS sample buffer, consisting of 30% glycerol, 3% SDS, 15 mM Na-EDTA, 2.0 M 2-mercaptoethanol, 0.3 mg/ml bromophenol blue, 0.375 M Tris-HCl (pH 6.8); the samples were boiled for 3 min before electrophoresis in 12.5% (except where noted) discontinuous polyacrylamide–SDS gels (Thomas and Kornberg, 1975). Assays of fraction II and the affinity-purified ligase system were performed under conditions of linearity, both in time and in enzyme.

Lysozyme was denatured by iodination, using the chloramine T method (Ciechanover et al., 1980). Histone H2B (Boehringer) was used as a ubiquitination substrate without prior chemical modification. We find that ... of histone H2B as a substrate has several technical advantages ... use of denatured lysozyme.

In Vivo Labeling with ^{35}S-Methionine, Acid Extraction, and Electrophoresis: Resolution of Free Ubiquitin and uH2A Semihistone in Whole-Cell Acid Extracts

Labeling medium was methionine-free MEM (Flow), supplemented with glutamine (0.29 g/l), additional glucose (3.5 g/l), 1× nonessential amino acid solution (GIBCO), sodium pyruvate (0.11 g/l), $Fe(NO_3)_2$ (300 nM), and 20 mM Na-HEPES (pH 7.5). Water baths were used for temperature control. Methionine depletion was by three washes in labeling medium for a total of ... hr with incubations at 3×10^5 cells per milliliter; cells were pelleted by ... ed centrifugation at either room temperature or 39°C, as appropri... each experimental point, 3×10^6 cells were labeled in a volume of ... μl with 65 μCi of ^{35}S-methionine (1420 Ci/mmole, Amersham). The pulse was terminated by immersion of the round-bottom sample tubes in ice water; the solutions were then transferred to Eppendorf centrifuge tubes and centrifuged at 12,800 × g for 30 sec. The supernatant was withdrawn, and the tube immersed in dry ice–acetone. The cell pellet was subsequently extracted with 0.5 N HCl at 4°C as previously described (Bonner et al., 1980), except that phenylmethylsulfonyl fluoride (PMSF, 0.5 mM) and protamine sulfate (2 mg/ml, Sigma) were included.

To resolve and detect both free ubiquitin and uH2A semihistone electrophoretic bands in a sample of whole-cell acid extract, under conditions ... many samples had to be analyzed, we developed the composite ... described below. The samples of whole-cell acid extracts ... ed for electrophoresis as described previously; Bonner et al., 1980), were loaded onto a composite gel containing 2 M urea, 0.4% Triton X-100, 0.9 M CH_3COOH, and 50 mM NH_4OH. The gel (1.5 mm thick; 25 cm total length) was polymerized with TEMED and ammonium persulfate, and preelectrophoresed with 0.9 M CH_3COOH, 50 mM NH_4OH as a running buffer at 150 V for 30–40 hr. For electrophoresis, the running buffer was 0.9 M CH_3COOH, 0.1 M glycine (Bonner et al., 1980). The stacking gel (3 cm long) was 4% acrylamide, 0.02% bisacrylamide, and did not contain Triton X-100. The upper portion of the separating gel (11 cm long) was 10% acrylamide, 0.19% bisacrylamide. The lower portion of the separating gel (11 cm long) was 25% acrylamide, 0.025% bisacrylamide. Electrophoresis was at 150 V until the dye (methyl green) reached the bottom of the 25% polyacrylamide gel. The lower half of the separating gel (25% polyacrylamide) was stained for 1 hr with several changes of 25% methanol, 1.8 M CH_3COOH, 5% formaldehyde, and 0.1% Coomassie brilliant blue R-250 and briefly destained. The use of formaldehyde was necessary to prevent losses of small proteins, particularly ubiquitin (data not shown). The location of the marker ubiquitin band was identified and marked, and the gel was subjected to the PPO-mediated fluorography as previously described (Bonner and Laskey, 1974). Fluorograms were analyzed by quantitative densitometry. Identification of the fluorographic band as ubiquitin was confirmed in separate experiments by two-dimensional gel electrophoresis (second dimension: 15% acrylamide, 0.1% bisacrylamide, 8 M urea, 0.9 M CH_3COOH, and no Triton X-100; data not shown).

The upper half of the separating gel (10% polyacrylamide) was transferred to a solution of 2 M Na-salicylate (Chamberlain, 1979), 5% glycerol, 0.1 M 2-mercaptoethanol, 70 mM CH_3COOH, and soaked for 30 min. A fluorogram of the wet gel after an overnight exposure at −70°C was then used to locate the uH2A band, which was excised as a single 7 mm wide strip containing the uH2A band and immediately adjacent protein bands from each lane (including the lanes of uH2A marker). This strip was incubated for 1 hr in 20% glycerol, 2% SDS, 0.65 M 2-mercaptoethanol, 0.1 mg/ml bromophenol blue, 0.125 M Tris-HCl (pH 6.8), followed by a second-dimension electrophoresis in an 18% polyacrylamide–SDS gel system described previously (Thomas and Kornberg, 1975). This fractionation scheme allowed fluorographic detection of both ubiquitin and uH2A in each electrophoresed sample.

To confirm that the residual intensity of the uH2A band observed in Figure 5A was due to uH2A, samples from an experiment similar to that of Figure 5 were prepared from salt-washed (0.3 M NaCl) nuclei by acid extraction (0.4 N H_2SO_4). These samples were analyzed by two-dimensional electrophoresis (first dimension, 15% polyacrylamide, acetic acid, urea, second dimension, 18% polyacrylamide–SDS).

Silver Staining of Proteins in Polyacrylamide–SDS Gels

After electrophoresis the gel (0.8 mm thick) was treated with the following solutions (30 min incubations): twice in 50% methanol; twice with 50% methanol, 2% formaldehyde; and six times with 50% methanol. The gel was then soaked for 20 min in a silver-staining solution (Oakley et al., 1980), rinsed three times in the course of 5 min in a 200-fold dilution of the staining solution (a modification introduced by Dr. F. Strauss in this laboratory), and developed according to Oakley et al. (1980).

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References

Andersen, M. W., Goldknopf, I. L., and Busch, H. (1981). Protein A24 lyase is an isopeptidase. FEBS Lett. *132*, 210–214.

Atidia, J., and Kulka, R. G. (1982). Formation of conjugates by ^{125}I-labeled ubiquitin microinjected into cultured hepatoma cells. FEBS Lett. *142*, 72–76.

Bonner, W. M., and Laskey, R. A. (1974). A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. *46*, 83–88.

Bonner, W. M., West, M. H. P., and Stedman, J. D. (1980). Two-dimensional gel analysis of histones in acid extracts of nuclei, cells, and tissues. Eur. J. Biochem. *109*, 17–23.

Brugge, J., Yonemoto, W., and Darrow, D. (1983). Interaction between the Rous sarcoma virus transforming protein and two cellular phosphoproteins: analysis of the turnover and distribution of this complex. Mol. Cell. Biol. *3*, 9–19.

Busch, H., and Goldknopf, I. L. (1981). Ubiquitin-protein conjugates. Mol. Cell. Biochem. *40*, 173–187.

Chamberlain, J. P. (1979). Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. Anal. Biochem. *98*, 132–135.

Chin, D. T., Kuehl, L., and Rechsteiner, M. (1982). Conjugation of ubiquitin to denatured globin is proportional to the rate of globin degradation in HeLa cells. Proc Nat. Acad. Sci. USA *79*, 5857–5861.

Choe, J., Kolodrubetz, D., and Grunstein, M. (1982). The two yeast histone H2A genes encode similar protein subtypes. Proc. Nat. Acad. Sci. USA *79*, 1484–1487.

Ciechanover, A., Hod, Y., and Hershko, A. (1978). A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes. Biochem. Biophys. Res. Commun. *81*, 1100–1105.

Ciechanover, A., Heller, H., Elias, S., Haas, A. L., and Hershko, A. (1980). ATP-dependent conjugation of reticulocyte proteins with the polypeptide required for protein degradation. Proc. Nat. Acad. Sci. USA *77*, 1365–1368.

Ciechanover, A., Elias, S., Heller, H., and Hershko, A. (1982). "Covalent" affinity purification of ubiquitin-activating enzyme. J. Biol. Chem. *257*, 2537–2542.

Ciechanover, A., Finley, D., and Varshavsky, A. (1984a). The ubiquitin-mediated proteolytic pathway and mechanisms of energy-dependent protein degradation. J. Cell. Biochem., in press.

Ciechanover, A., Finley, D., and Varshavsky, A. (1984b). Ubiquitin dependence of selective protein degradation demonstrated in the mammalian cell cycle mutant ts85. Cell *37*, 57–66.

Courtneidge, S. A., and Bishop, J. M. (1982). Transit of pp60$^{v\text{-}src}$ to the plasma membrane. Proc. Nat. Acad. Sci. USA *79*, 7117–7121.

Gavilanes, J. G., deBuitrago, G. G., Perez-Castells, R., and Rodriguez, R. (1982). Isolation, characterization, and amino acid sequence of a ubiquitin-like protein from insect eggs. J. Biol. Chem. *257*, 10267–10270.

Glover, C. V. C. (1982). Heat-shock effects on protein phosphorylation in Drosophila. In Heat Shock, M. J. Schlesinger, M. Ashburner, and A. Tissieres, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory), pp. 227–234.

Goldknopf, I. L., Tayler, C. W., Baum, R. M., Yeoman, L. C., Olson, M. O. J., Prestayko, A. W., and Busch, H. (1975). Isolation and characterization of protein A24, a "histone-like" non-histone chromosomal protein. J. Biol. Chem. *250*, 7182–7187.

Goldstein, G., Scheid, M., Hammerling, U., Boyse, E. A., Schlesinger, D. H., and Niall, H. D. (1975). Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. Proc. Nat. Acad. Sci. USA *73*, 11–15.

Haas, A. L., and Rose, I. A. (1982). The mechanism of ubiquitin activating enzyme. J. Biol. Chem. *257*, 10329–10337.

Haas, A. L., Warms, J. V. B., Hershko, A., and Rose, I. A. (1982). Ubiquitin activating enzyme. J. Biol. Chem. *257*, 2543–2548.

Hershko, A. (1983). Ubiquitin: roles in protein modification and breakdown. Cell *34*, 11–12.

Hershko, A., and Ciechanover, A. (1982). Mechanisms of intracellular protein breakdown. Ann. Rev. Biochem. *51*, 335–364.

Hershko, A., Heller, H., Ganoth, D., and Ciechanover, A. (1978). Mode of degradation of abnormal globin chains in rabbit reticulocytes. In Protein Turnover and Lysosome Function, H. L. Segal and D. J. Doyle, eds. (New York: Academic Press), pp. 149–169.

Hershko, A., Ciechanover, A., Heller, H., Haas, A. L., and Rose, I. A. (1980). Proposed role of ATP in protein breakdown: conjugation of proteins with multiple chains of the polypeptide of cofactor of ATP-dependent proteolysis. Proc. Nat. Acad. Sci. USA *77*, 1783–1786.

Hershko, A., Eytan, E., Ciechanover, A., and Haas, A. L. (1982). Immunochemical analysis of the turnover of ubiquitin-protein conjugates in intact cells. J. Biol. Chem. *257*, 13964–13970.

Hershko, A., Heller, H., Elias, S., and Ciechanover, A. (1983). Components of the ubiquitin-protein ligase system. J. Biol. Chem. *258*, 8206–8214.

Hightower, L. E. (1980). Cultured animal cells exposed to amino acid analogs or puromycin rapidly synthesize several polypeptides. J. Cell. Physiol. *102*, 407–427.

Hightower, L. E., and Smith, M. D. (1978). Effects of canavanine on protein metabolism in Newcastle disease virus-infected and uninfected chicken embryo cells. In Negative Strand Viruses and the Host Cell, B. W. J. Mahy and R. D. Barry, eds. (New York: Academic Press), pp. 395–405.

Kelley, P. M., and Schlesinger, M. J. (1978). The effect of amino acid analogues and heat shock on gene expression in chicken embryo fibroblasts. Cell *15*, 1277–1286.

Levinger, L., and Varshavsky, A. (1980). High-resolution fractionation of nucleosomes: minor particles, "whiskers" and separation of mononucleosomes containing and lacking A24 semihistone. Proc. Nat. Acad. Sci. USA *77*, 3244–3248.

Levinger, L., and Varshavsky, A. (1982). Selective arrangement of ubiquitinated and D1 protein-containing nucleosomes within the Drosophila genomes. Cell *28*, 375–385.

Marunouchi, T., Yasuda, H., Matsumoto, Y., and Yamada, M. (1980). Disappearance of a chromosomal basic protein from cells of a mouse temperature-sensitive mutant defective in histone phosphorylation. Biochem. Biophys. Res. Commun. *95*, 126–131.

Marunouchi, T., Mita, S., Matsumoto, Y., and Yasuda, H. (1983). A temperature-sensitive mutant and the nature of G2-mitosis transition. In Premature Chromosome Condensation. P. N. Rao, R. T. Johnson, and K. Sperling, eds. (New York: Academic Press), pp. 195–206.

Matsui, S., Seon, B. K., and Sandberg, A. A. (1979). Disappearance of a structural chromatin protein A24 in mitosis: implications for molecular basis of chromatin condensation. Proc. Nat. Acad. Sci. USA *76*, 6386–6390.

Matsui, S., Sandberg, A. A., Negoro, S., Seon, B. K., and Goldstein, G. (1982). Isopeptidase: a novel eukaryotic enzyme that cleaves isopeptide bonds. Proc. Nat. Acad. Sci. USA *79*, 1535–1539.

Matsumoto, Y., Yasuda, H., Mita, S., Marunouchi, T., and Yamada, M. (1980). Evidence for the involvement of H1 histone phosphorylation in chromosome condensation. Nature *284*, 181–183.

Matsumoto, Y., Yasuda, H., Marunouchi, T., and Yamada, M. (1983). Decrease in uH2A (protein A24) in a mouse temperature-sensitive mutant. FEBS Lett. *151*, 139–142.

Mita, S., Yasuda, H., Marunouchi, T., Ishiko, S., and Yamada, M. (1980). A temperature-sensitive mutant of cultured mouse cells defective in chromosome condensation. Exp. Cell Res. *126*, 407–416.

Oakley, B. R., Kirsh, D. R., and Morris, N. R. (1980). A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal. Biochem. *105*, 361–363.

Rose, I. A., and Warms, J. V. B. (1983). An enzyme with ubiquitin carboxy-terminal esterase activity from reticulocytes. Biochemistry *22*, 4234–4237.

Seale, R. L. (1981). Rapid turnover of the histone–ubiquitin conjugate, protein A24. Nucl. Acids Res. *9*, 3151–3158.

Thomas, J. O., and Kornberg, R. D. (1975). An octamer of histones in chromatin and free in solution. Proc. Nat. Acad. Sci. USA *72*, 2626–2630.

Varshavsky, A., Levinger, L., Sundin, O., Barsoum, J., Ozkaynak, E., Swerdlow, P., and Finley, D. (1983). Cellular and SV40 chromatin: replication, segregation, ubiquitination, nuclease-hypersensitive sites, HMG-containing nucleosomes and heterochromatin-specific protein. Cold Spring Harbor Symp. Quant. Biol. *47*, 511–528.

...est, M. H. P., and Bonner, W. M. (1980). Histone 2b can be modified by attachment of ubiquitin. Nucl. Acids Res. *8*, 4671–4680.

Wilkinson, K. D., Urban, M. K., and Haas, A. L. (1980). Ubiquitin is the ATP-dependent proteolysis factor I of rabbit reticulocytes. J. Biol. Chem. *255*, 7529–7532.

Wu, R. S., Kohn, K. W., and Bonner, W. M. (1981). Metabolism of ubiquitinated histones. J. Biol. Chem. *256*, 5916–5920.

Yasuda, H., Matsumoto, Y., Mita, S., Marunouchi, T., and Yamada, M. (1981). A mouse temperature-sensitive mutant defective in H1 histone phosphorylation is defective in deoxyribonucleic acid synthesis and chromosome condensation. Biochemistry *20*, 4414–4419.